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European Journal of Pharmacology 408 (2000) 227–232

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Amiodarone has exclusively non-genomic action on cardiac β-adrenoceptor regulation

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Received 14 July 2000; received in revised form 11 October 2000; accepted 16 October 2000

Abstract

The antiarrhythmic drug amiodarone down-regulates the density of cardiac β-adrenoceptors behaving as a triiodothyronine (T_3) antagonist. It is still unclear if amiodarone acts at the nuclear (genomic) and/or the non-genomic levels. Using Northern blot analysis, we showed that the amiodarone had no effect on the increase of β_1-adrenoceptor mRNA level induced by the T_3-administration in the heart of thyroidectomised rats. Thus, our results suggest that amiodarone has no genomic effect. Consequently, we investigated whether amiodarone down-regulation of β-adrenoceptor number in T_3-stimulated cardiomyocytes could be explained by changes in the rate of cell surface receptor protein turnover. Indeed, the binding studies of cyclohexidemide-treated cells showed that amiodarone suppressed the T_3-induced decrease in the rate of the cell surface receptor disappearance. In conclusion, our findings indicate that the modulation of cardiac β-adrenoceptor density by amiodarone involves only non-genomic targets required in T_3-dependent regulation of the cell surface β-adrenoceptor turnover.

Keywords: Amiodarone; β-Adrenoceptor turnover; Triiodothyronine; Cardiomyocyte

1. Introduction

Amiodarone is a highly effective drug against a wide range of cardiac arrhythmia's (Podrid, 1995; Wiersinga, 1997). Some of the electrophysiological effects of amiodarone are due to its antiadrenergic action. Amiodarone has no affinity to β-adrenoceptor and does not affect basal or isoproterenol-increased intracellular cAMP levels (Drvota et al., 1999). In contrast, it down-regulates the cardiac β-adrenoceptor density as reported in several studies in vivo and in vitro (Drvota et al., 1999; Hartong et al., 1990; Perret et al., 1992; Wiersinga, 1997; Yin et al., 1992, 1994). This effect is due rather to an antagonistic effect on triiodothyronine (T_3) than to a direct action. Indeed, the amiodarone has no effect on receptor number when given to hypothyroid animals (Yin et al., 1992) whereas it reduced the T_3-induced increase in β-adrenoceptor density in vivo (Hartong et al., 1990; Yin et al., 1992) and in vitro (Yin et al., 1992). These findings and the structural similarities between amiodarone and T_3 (Chalmers et al., 1992) argue for a possible antagonism between them.

However, the mechanism of amiodarone effect on β-adrenoceptors is still unclear. Since the drug behaves mainly as a T_3 antagonist, it was proposed to have, like T_3 (Davis and Davis, 1997; Vassy et al., 1997), two possible targets, nuclear (genomic) and extranuclear (non-genomic) ones (Wiersinga, 1997; Yin et al., 1994). The T_3 forms a complex with specific nuclear receptor and binds to thyroid hormone response elements located in the promoter region of β_1-adrenoceptor (the major β-adrenoceptor subtype in heart) gene (Collins et al., 1993) thus stimulating the transcription and increasing the specific mRNA level (Bahouth, 1991). Since amiodarone has been shown to inhibit the T_3 binding to thyroid hormone receptors (Drvota et al., 1995a), it is theoretically possible that amiodarone could modulate the transcriptional effects described above. Up to date, there are no direct experimental evidences for this hypothesis. Concerning the non-genomic mechanism, we have recently shown that an early increase of β-adren-

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00793-7

oceptor density in response to T_3 is independent of protein synthesis (Vassy et al., 1997). In the present study, we explored the effect of amiodarone on β_1-adrenoceptor mRNA level in the heart of hypothyroid rats treated or not with T_3, amiodarone or both, T_3 and amiodarone. In addition, we have evaluated if amiodarone is able to modulate this non-genomic T_3 action and we found that the drug decreased the cell surface density of β-adrenoceptors mainly by increasing the rate of receptor disappearance.

2. Materials and methods

2.1. Animals

Twenty thyroidectomised male rats (150 g of weight) purchased from Iffa Credo (Lyon, France) were given 0.9% $CaCl_2$ and 0.03% methimazole in their drinking water during 7 weeks. The rats were housed in a temperature controlled room (22 ± 1°C) and were fed on stock diet (U.A.R., Epinay sur Orge, France). Then, the animals were divided in four groups: control (saline-treated), amiodarone-treated, saline + T_3-treated and amiodarone + T_3-treated. All treatments were accomplished daily by gastric intubation. Amiodarone (Sigma, St. Louis, MO, USA) was administered (50 mg/kg) for 8 days. This duration of amiodarone treatment was necessary because of low oral bioavailability (Hartong et al., 1990; Mason, 1987) and slow myocardial concentration (Mason, 1987). The T_3 (Sigma) was administered (0.5 mg/kg) daily during 3 days. In these experimental conditions, we observed that T_3 up-regulated the cardiac β-adrenoceptor density and amiodarone down-regulated the number of β-adrenoceptors but only in the presence of T_3 (Yin et al., 1992; Perret et al., 1992). The effects of different treatments on thyroid status were assessed by measurements of serum-free T_3 using commercial radioimmunoassay kit (Behring, Marburg, Germany). After treatment, the animals were decapitated and their cardiac ventricles were rapidly dissected and stored at −80°C.

2.2. Northern blot analysis

Total RNA was isolated from homogenates of rat cardiac ventricles using RNA plus solution (Bioprobe Systems, Montreuil-sous-Bois, France). Its concentration was determined by spectrometry at 260 nm. Fifty microgram of total RNA were electrophoresed in 1.2% agarose formaldehyde-denaturing gel and transferred to a nylon membrane (Hybond N, Amersham, United Kingdom) overnight in 10 × SSC (0.15 M NaCl + 0.15 M sodium citrate), then fixed by baking at 80°C for 2 h. The blot was prehybridized in 50% formamide, 5 × SSPE (saline-sodium phosphate-EDTA), 5 × Denhart's solution, 0.5% sarcosyl and 125 μg/ml heat-denatured salmon sperm DNA at 42°C for at least 3 h. Then, the blot was hybridised with a [^{32}P]-labelled specific probe and cyclophilin cDNA probe. The rat β_1-andrenoceptor probe 896 bp *Pst*I DNA (Mashida et al., 1990) kindly provided by Dr. Curtis A. Mashida (Oregon Regional Primate Research Center, OR, USA) and rat 700 bp cDNA cyclophilin probe (Danielson et al., 1988) generously offered by Dr. James Douglas (Oregon Health Sciences University Portland, OR, USA) were labeled by random priming using [α-^{32}P]dCTP and multiprime labeling kit (both from Amersham). Blots were washed twice in 1 × SSC-0.5% SDS, one at room temperature and other at 65°C, followed by washing in 1 × SSC-0.1% SDS at 65°C, and subjected to autoradiography. Specific band densities were quantified by densitometry (Biocom SA, Les Ulis, France). The amount of total RNA in each sample was internally standardised within each blot by correcting the specific mRNA levels according to the levels of cyclophilin mRNA.

2.3. Cardiomyocyte isolation and culture

Monolayer cultures of chick embryonic ventricular beating cells were prepared as previously described (Marsh et al., 1982). Briefly, 11-day-old chick-embryonic hearts were removed and the ventricular fragments were dissociated by repeated cycles of incubation with 8.5% porcine pancreatic trypsin (Biosys, Compiègne, France). The resulting suspension was adjusted to 3×10^5 cells/ml in medium 199 with Hanks' salt (Eurobio, Paris, France) supplemented with 5% foetal calf serum (Gibco, Paris, France) and placed (2 ml/well) in 6-well tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, USA). The cells were incubated for 48 h in a humidified 5% CO_2/95% air atmosphere at 37°C to allow their attachment. Then, the medium was replaced by an insulin-containing synthetic serum-replacement medium (SSR2, Medi-cult, Denmark) supplemented with 10^{-8} M testosterone (Sigma, France). The proteins or thyroid hormones were not detected in this synthetic medium, whereas 10^{-13} M free T_3 was found in the 5% foetal calf serum-containing medium. In contrast to the calf serum supplemented-medium, the SSR2 medium prevented the proliferation of the non-contractile fibroblasts. Under these conditions, more than 80% of the cells were able to contract when stimulated by an electric impulse (Yin et al., 1992) and expressed desmin, a marker of muscular cells (Vassy et al., 1997). The cell treatment began 12 h after the medium replacement. T_3 and amiodarone (both from Sigma) were stored as 10^{-2} M solution in 26 mM NaOH and dimethyl sulfoxide, respectively, at −20°C in the dark. The stock solutions were diluted with sterile water to the required concentrations before use. The time-course experiments were performed in such a way that all incubations ended at the same time. It was possible

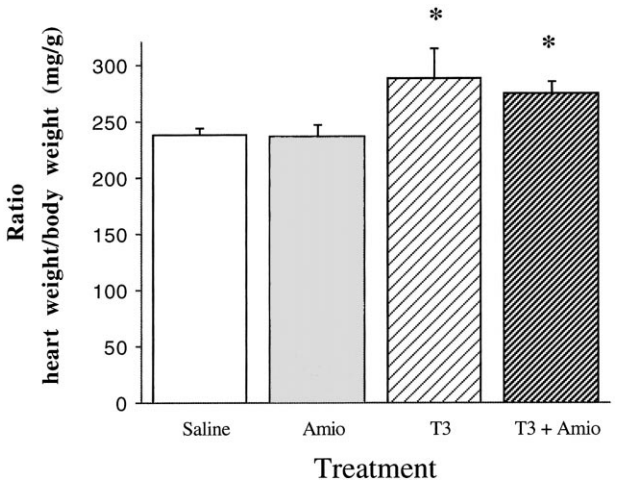

Fig. 1. Effect of amiodarone (Amio), T_3 and Amio + T_3 on heart weight of thyroidectomised rats. Amiodarone was administered (50 mg/kg) daily by gastric intubation during 8 days. The T_3 was administered (0.5 mg/kg) daily during 3 days. After treatment, the animals were decapitated and their hearts were rapidly dissected and weighted. Each experimental group contained five animals. $^{*}P < 0.05$ as compared to control (saline) group.

because the β-adrenoceptor number remained stable for the 72 h of incubation in the SSR2 medium.

2.4. Determination of β-adrenoceptor density

Cell surface β-adrenoceptor were measured as already described (Delavier-Klutchko et al., 1984) by using [^{3}H]CGP 12177, (−)-4-(3-tertiary-butylamino-2-hydroxy-propoxy)-(5,7-^{3}H)benzimidazol-2-one (38 Ci/mmol; Amersham, Paris, France). After incubation in different conditions, the cells were rinsed 4 times with cold (4°C) Hank's balanced salt solution containing 2×10^{-5} M HEPES and 0.1% bovine serum albumin, pH 7.4 (HHSA). Binding was initiated by the addition of [^{3}H]CGP 12177 at the increasing concentrations (0.06 to 2 nM) in 1 ml HHSA. After 60 min of incubation at 37°C, the reaction was stopped by washing the wells 4 times with 2 ml of cold HHSA. Then, the cells were solubilized with 1 ml of 0.2 N NaOH and the radioactivity of the solution was determined in a scintillation counter (Beckman). Non-specific binding was defined as the amount of radioactivity remaining in the presence of 2×10^{-6} M (±)-propranolol and accounted for 5–20% of the total binding. Binding affinity and density were calculated by Scatchard analysis. B_{max} was expressed as the number of sites/cell.

2.5. Statistics

Data are presented as the means ± SEM. Analysis of variance followed by the Scheffe's test was used to compare the means of the different parameters of each group. A two-tailed test P value < 0.05 was considered to reflect a statistically significant difference.

3. Results

3.1. Effect of amiodarone on heart weight

As expected, T_3-administration increased by 21% the relative heart weight, thus evidencing the efficiency of thyroid hormone treatment. In contrast, the amiodarone treatment did not affect significantly the relative weight of heart neither in the thyroidectomised control rats nor in the T_3-treated animals (Fig. 1).

3.2. Effect of amiodarone on total RNA and β_1-adrenoceptor mRNA level

The total RNA quantity contained in 1 mg of heart (1.2 ± 0.17 μg; $n = 20$ was not significantly changed nei-

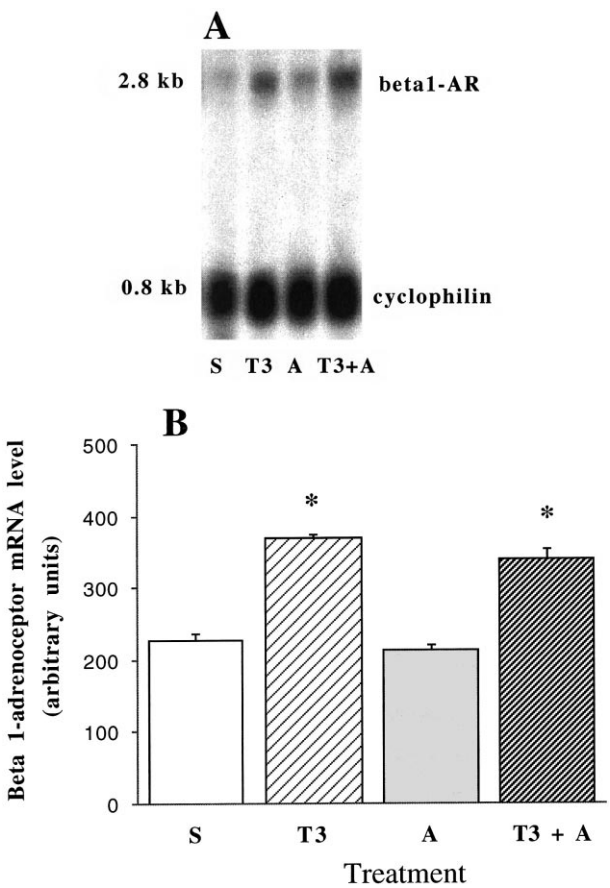

Fig. 2. Effect of amiodarone (A), T_3 and A + T_3 on β_1-adrenoceptor mRNA level in heart of thyroidectomised rats. Amiodarone was administered (50 mg/kg) daily by gastric intubation during 8 days. The T_3 was administered (0.5 mg/kg) daily during 3 days. The control group was treated with saline (S) for 8 days. After treatment, the animals were decapitated, their hearts rapidly dissected and total RNA isolated. β_1-adrenoceptor and cyclophylin mRNA levels were determined by Northern blot analysis (A). Then, the specific band densities were quantified by densitometry. The amount of total RNA in each sample was internally standardised within each blot by correcting the specific mRNA levels according to the levels of cyclophilin mRNA (B). Each experimental group contained five animals and each bar represents the mean of five determinations. $^{*}P < 0.05$ as compared to control S group.

ther by amiodarone nor by T_3 nor by two agent combined-treatment. The specific β_1-adrenoceptor mRNA level was 1.7-fold increased following the T_3-administration but not significantly altered by the amiodarone treatment of the thyroidectomised control rats as well as the T_3-treated animals (Fig. 2). It is noteworthy that, in the experimental conditions used in this study, the amiodarone down-regulates the number of T_3-increased β-adrenoceptor level (Yin et al., 1992; Perret et al., 1992).

3.3. Effects of amiodarone on cell surface β-adrenoceptor density is time-dependent

We have performed a time course of amiodarone effect in cardiomyocytes (Fig. 3). In this experimental model in vitro, the supply of amiodarone to cardiac cells is direct and immediate, contrary to in vivo model in which the myocardial concentration of drug increases slowly (Holt et al., 1983; Mason, 1987). As amiodarone only acts on T_3-induced β-adrenoceptors (Yin et al., 1992, 1994) the cells were pretreated with T_3 10^{-8} M for 48 h. In these conditions, the increase of β-adrenoceptor level reaches a plateau (Vassy et al., 1997). As expected, the level of β-adrenoceptors was significantly higher on T_3-treated cells compared to control non-pretreated cells. Then, the amiodarone was added. In the case of T_3-pretreated cardiomyocytes, the significant and time-dependent inhibitory effect was detected from 5 h of amiodarone administration. During 24-h treatment, the amiodarone induced the loss of 5980 binding sites/cell. In contrast, no significant changes in the β-adrenoceptor density was observed on control not

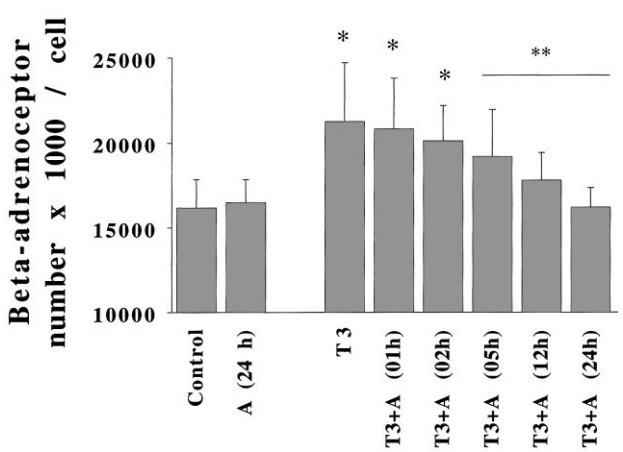

Fig. 3. Time course of amiodarone 10^{-6} M (A) effect on β-adrenoceptor number in cardiomyocytes preincubated (T_3) or not (control) with T_3 10^{-8} M for 48 h. Cell surface β-adrenoceptors were measured by binding studies using [^{3}H]CGP 12177. Non-specific binding was defined as the amount of radioactivity remaining in the presence of 2×10^{-6} M (±)-propranolol. Binding density was calculated by Scatchard analysis. B_{max} was expressed as the number of sites/cell. Each point represents the mean of five determinations. $^{*}P < 0.05$ as compared to control cells at 0 h of amiodarone treatment. $^{**}P < 0.05$ as compared to T_3-pretreated cells at 0 h of amiodarone treatment.

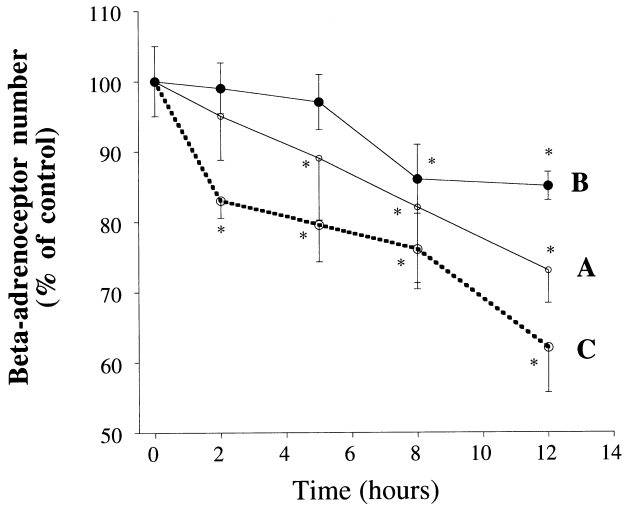

Fig. 4. Determination of β-adrenoceptor disappearance rate. The cardiomyocytes were incubated with the protein synthesis inhibitor cycloheximide (2×10^{-5} M) in the absence (A) or presence of T_3 10^{-8} M (B) or T_3 10^{-8} M + amiodarone 10^{-6} M (C). Cell surface β-adrenoceptors were measured by binding studies using [^{3}H]CGP 12177. Non-specific binding was defined as the amount of radioactivity remaining in the presence of 2×10^{-6} M (±)-propranolol. Binding density was calculated by Scatchard analysis. The β-adrenoceptor density is expressed as the percent of binding sites in untreated cells. Each point represents the mean of five determinations. $^{*}P < 0.05$ as compared to control.

T_3-pretreated cells as previously reported by our laboratory (Yin et al., 1994).

3.4. Effect of amiodarone on disappearance of β-adrenoceptors on surface of T_3-pretreated cardiomyocytes

To explore the mechanism of amiodarone action on T_3-treated cells, we have studied the disappearance rate of cell surface β-adrenoceptors using cycloheximide, a protein synthesis inhibitor (Fig. 4). Until 12 h of administration at the concentration used, the cycloheximide did not affect either cardiomyocyte number or total protein content (data not shown). In the presence of cycloheximide, the synthesis of β-adrenoceptors is suppressed and the time course of the cell surface density of receptors allows to estimate their rate of disappearance in three different conditions as follows: (A) $2.25 \pm 0.13\%/h$ for cycloheximide-treated cells; (B) $1.25 \pm 0.58\%/h$ for cycloheximide + T_3-treated cells and (C) $3.17 \pm 0.47\%/h$ for cycloheximide + T_3 + amiodarone-treated cells. Thus, amiodarone was able to enhance significantly the T_3-induced decrease in disappearance rate of β-adrenoceptors on cardiomyocytes.

4. Discussion

In this work, we evaluated for the first time, to our knowledge, the effect of amiodarone on the β_1-adrenoceptor mRNA level in heart. We have studied the β_1-subtype

since it represents the predominant (more than 80%) population of ventricular cell β-adrenoceptors (Bahouth, 1991; Drvota et al., 1995b). Moreover, T_3 increases the level of β_1-adrenoceptor mRNA and protein but does not affect this of β_2-subtype messenger nor protein (Bahouth, 1991). It suggests that if amiodarone has a T_3-antagonizing effect on specific mRNA level it should be detected using Northern blot analysis of β_1-subtype mRNA. Nevertheless, data reported here demonstrate that amiodarone does not regulate the specific mRNA level. These results agree with our findings that amiodarone is able to block completely the early phase of T_3 effect on β-adrenoceptor number but only partially the late one (Vassy et al., 1994). As we have previously (Vassy et al., 1997) proposed, the early phase corresponds to non-genomic action of T_3 and the late one is the resultant of both, non-genomic and genomic, effects of thyroid hormone. Furthermore, the genomic action of T_3 requires a lag period of 6–10 h elapsed before an appreciable change in the number of β_1-adrenoceptors per cell was observed (Bahouth, 1991), whereas the amiodarone-induced down-regulation of receptors was detected as soon as 5 h after the treatment began. All these data strongly suggest that the mechanism of β-adrenoceptor number reduction by amiodarone does not involve genomic events.

We postulate that amiodarone has only post-translational action in cardiomyocytes since the drug effect on β-adrenoceptor density is cycloheximide-independent as we observed in the present study. Further, we demonstrate here that amiodarone treatment increased the T_3-reduced disappearance rate of cell surface β-aderenoceptor protein. It suggests that modifications in receptor protein disappearance rate from cell surface is the underlying mechanism. The changes in protein degradation induced by thyroid hormones in heart atria and ventricles were observed by Canavan et al. (1994). We strongly support for the first time, to our knowledge, the amiodarone intervention in this kind of protein regulation.

Although it is clear now that amiodarone acts only in the presence of T_3 in an antagonising manner, it is difficult to speculate if thyroid hormone and drug compete for the same target. It could also be probable that T_3 activates an unknown pathway leading to slowing of the β-adrenoceptor turnover rate and that amiodarone inhibits some step(s) along this pathway. Further studies are necessary to explain this new mechanism of T_3 and the amiodarone action on myocardial β-adrenoceptors.

This inhibitory effect of amiodarone seems specific to β-adrenoceptors since we observed that drug did not antagonise the T_3-induced increase in the heart weight which is mainly due to a global augmentation of protein synthesis (Canavan et al., 1994). Moreover, this effect is tissue-specific because amiodarone has no effect on T_3-induced increase in β_1-adrenoceptor density in brown adipocytes, as recently reported by our laboratory (Adli et al., 1999).

In conclusion, our results show that the downregulation of myocardiac β-adrenoceptor density by amiodarone does not involve changes in the specific mRNA level but it is due to modifications of the rate of cell surface receptor disappearance. This study indicates a new pharmacological mechanism for tissue-specific control of β-adrenoceptors by alteration of protein turnover rate.

Acknowledgements

We are grateful to Drs. Curtis A. Mashida and James Douglas for providing generously the rat β_1-adrenoceptor probe 896 bp *Pst*I DNA and rat 700 bp cDNA cyclophilin probe, respectively.

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